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14. ABSTRACT We have described a new form of epistasis in which direct, long range, physical interactions between genes, or gene-gene interactions mediated by specialized DNA binding proteins such as CTCF, lead to modification of phenotypic read-out. Using the associated chromatin trap (ACT) and chromosome conformation capture (3C) assays which are designed to assess physical propinquity, we investigated long range interactions of the human NF1 gene that are mediated by CTCF in normal cultured cells and in cells derived from patients with neurofibromatosis. Among the genes that were physically associated with NF1 (which is on chromosome 17) was ARF4 (ADP-ribosylation factor 4, a member of the RAS superfamily involved in membrane traffic, signal transduction and organelle integrity on chromosome 3p14.3. The relative expression of ARF4 was increased in cells and tissues from patients with neurofibromatosis compared to normal cells, suggesting that the interchromosomal interactions of NF1 regulate gene expression on chromosome 3p14.3. 4. Data obtained this year suggests that ARF4 might play a role in neurofibromatosis 1 tumorigenesis. The search for novel remote gene interactions with NF1 promises to open up totally new ranges of therapeutic targets.

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INTRODUCTION

One of the most remarkable aspects of neurofibromatosis 1 is the great variability in the expression of the disease, in which some affected patients may have few or mild manifestations, while others may have quite severe disease. Epistasis refers to a gene interaction in which gene A interferes with the phenotypic expression of gene B, in such a way that even if gene B is the "disease gene" (e.g., *NF1*), gene A may play an important or determining role in how the disease is manifest. We have described a new form of epistasis in which direct, long range, physical interactions between genes, or gene-gene interactions mediated by specialized DNA binding proteins such as CTCF, lead to modification of phenotypic read-out.(1)

BODY

***Task 1/2*: Characterize interactions between *NF1* and *IGF2* in normal and tumor cells.**

In our previous work, we had shown that the mouse *Nf1* gene interacted with *Igf2* (2). As we reported in the Annual Report for 2006-2007, we confirmed this association in humans, demonstrating by chromosome conformation capture (3C) and FISH (**Figure 1**) that the imprinting control region between *IGF2* and *H19* on chromosome 11 interacted with *NF1* on chromosome 17.

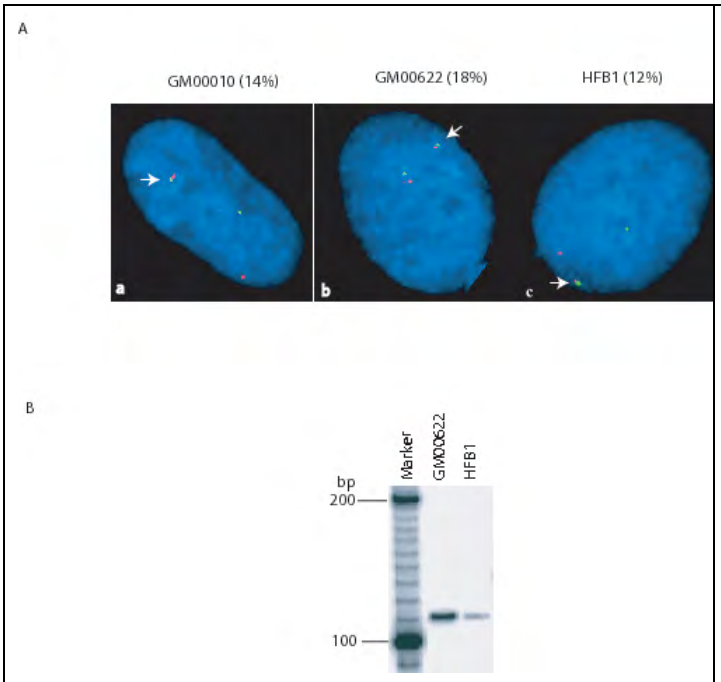

Figure 1. Interchromosomal association of NF1 gene with ARF4 gene region.

A. FISH results from two normal human fibroblast cell lines (HFB1 and GM00010) and a cell line derived from the unaffected skin of a patient with neurofibromatosis (GM00622). There was co-localization of the NF1 locus (green) on chromosome 17 and the ARF4 locus (red) on chromosome 3. Two hundred nuclei were counted in each cell line. Co-localization was demonstrated in 12-18% of cells counted. Co-localized signals are indicated by white arrows.

B. Chromosome conformation capture (3C) assay in HFB1 and GM00622 cell lines. 3C results confirm the association between chromosomes 17q11.2 and 3p14.3. The 11-bp amplified band consists of the re-ligated chimeric DNA fragments containing segments from both the NF1 and the ARF4 regions.

We decided to see what happened to long range interactions in cells in which *IGF2* imprinting is lost; in these cells, the interaction between *IGF2* and *NF1* is also lost. We applied the ACT assay using *GF@* as bait. *IGF2* interacts with TSSC4 (tumor suppressing subchromosomal transferable fragment candidate gene 4) and TSSC6, (**Fig. 2, clone G2-9**), a non-imprinted gene in the 11p15 imprinted region upstream of *IGF2*. This was determined independently in two cell lines, using traditional 3C in one line and ACT in another line. However, in a cancer cell line lacking *IGF2* imprinting, there was no association of the *IGF2* gene with this putative tumor suppressor gene. As shown in **Fig. 1**, the ACT assay yields different interactions in the normal cells compared with the LOI cancer cell line. While many more controls need to be done, this study suggests that changes in long range interactions, some of which involve putative tumor suppressor genes like *NF1*, occur in cancer cells.

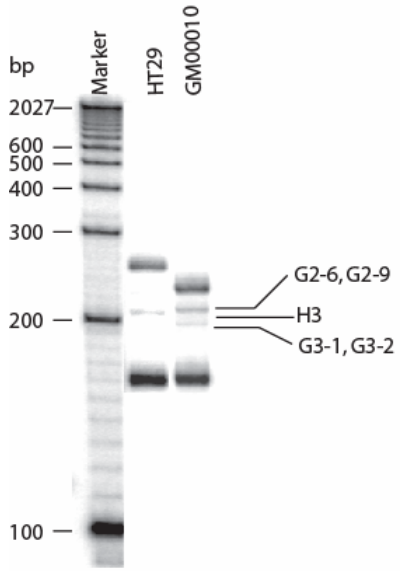

Figure 2. ACT assay of human *IGF2* (near promotors 2 and 3) in colon cancer HT29 cell line and normal fibroblast cell line GM00010. Msp1-adaptor-ligated 3C DNA was amplified using specific sets of primer pairs. Clone H3 is located at chromosome 8q21.2 near *REX01L2P*. Clone G2-6 is at chromosome 11p15.5 near *TSSC6*, *TSSC4*, *CD81* and *TSPAN* 32. Clone G3-1 is at chromosome 11p15.5 between *IGF2* and *INS*. Clone G3-2 is at chromosome 13q14.11.

Task 3: Search for new *NF1*-interacting partners

Using the ACT assay, we began our exploration of which other genes interacted with *NF1* in both normal cell lines and in cell lines derived from patients with neurofibromatosis. Using several CTCF-binding ECR regions, we have elucidated many of these interacting genes, which are located on the multiple different chromosomes. As we reported last year, we became particularly interested in the interaction of *NF1* and *ARF4* (ADP-ribosylation factor 4, a member of the RAS superfamily involved in membrane traffic, signal transduction and organelle integrity). We confirmed the ACT data which suggested a physical interaction by directly demonstrating the interaction of one *NF1* allele with one *ARF4* allele using FISH analysis.

We have expanded on the data presented in the previous Annual Report that had shown an increase in *ARF4* mRNA in several cell lines derived from patients with neurofibromatosis, suggesting that *ARF4* may play a role in the manifestations of the disease. We confirmed that *ARF4* was elevated in 20/23 tumor tissue samples from patients with neurofibromatosis, further implicating this gene in tumorigenesis in this disease. *ARF4* expression was always higher in tissues derived from patients with severe manifestations of neurofibromatosis than in tissues derived from normal individuals or from patients with less severe or atypical findings.

Since methylation of the CTCF binding site may restrict CTCF binding and thereby modify the three-dimensional architecture, we assessed DNA methylation at the CTCF binding site on chromosome 3p14.3 near *ARF4*. The CTCF binding site is >90% methylated in a normal human cell line, but the degree of DNA methylation was substantially lower in the

neurofibromatosis-derived cell lines. The amount of unmethylated DNA was increased 5-10 times compared to control in each of five *NF1* cell lines, although the extent of DNA methylation did not correlate with *ARF4* expression in these cells. The CTCF binding site near *ARF4* at 3p14.3 was completely methylated in the normal skin samples, but was unmethylated to varying degrees in all of the neurofibromatosis samples

When normal (GM00010) and neurofibromatosis-derived cells (GM01633) were treated with the DNA methylation inhibitor 5-azacytidine, DNA methylation decreased at the CTCF binding site at the *ARF4* locus, and expression of *ARF4* increased, compared to control cells. (**Figure 3**). However, NF1 gene expression decreased dramatically after 5-azacytidine treatment. Since the CTCF binding region of ECR4 (chromosome 17q11.2) was initially unmethylated, 5-azacytidine treatment should not have altered its methylation status. Decreased NF1 expression may have been caused by epigenetic changes in other regions near NF1 or by changes in long-range interactions.

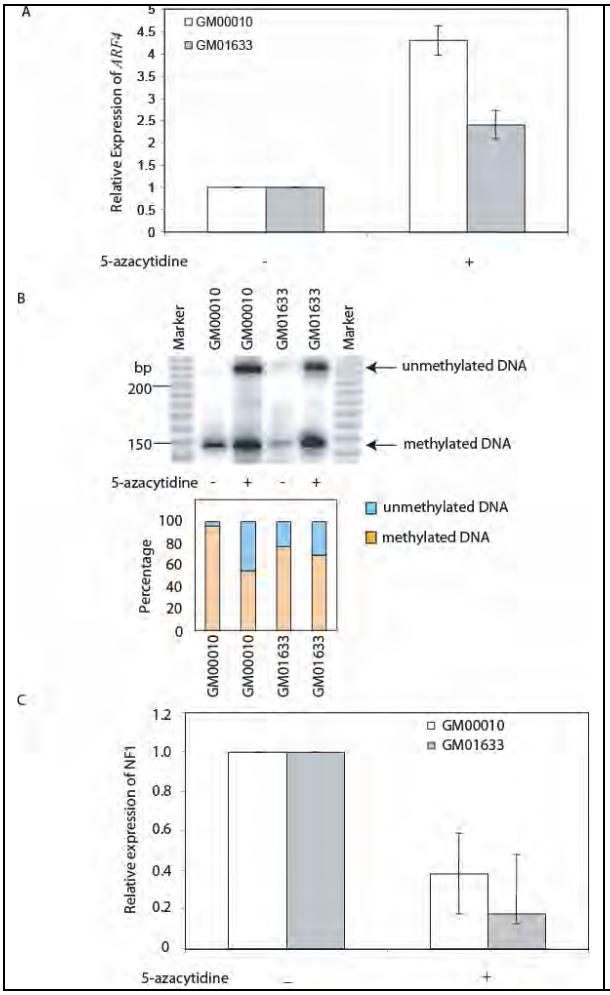

Figure 3. Treatment of cell lines with 5-azacytidine

A. Q-PCR of ARF4 in control (GM00010) and neurofibromatosis-patient derived (GM01633) cell lines. Cells were treated with 5 µM of 5-azacytidine for 48 hours (Sigma-Aldrich, St. Louis, MO, USA). n = 4.

B. Methylation status of CTCF binding site at the ARF4 locus. Sodium bisulfite-treated genomic DNA of GM00010 and GM01633 were used to amplify the CTCF binding region; PCR products were subjected to digest by Aci I, and semi-quantification of methylated DNA and unmethylated DNA was performed. Unmethylated DNA at the CTCF binding site in 5-azacytidine-treated increased in both cell lines.

C. NF1 expression was measured by real-time PCR with primers 5260 and 5261; L7, which was amplified by primers 1266 and 1267, acted as a normalizing control. n=4.

A potential functional relationship between *ARF4* and *NF1* is shown in **Figure 4**. ARF4 binds to the intracellular portion of the EGFR and stimulates PLD2 activity. PLD2 catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline, and PA then recruits Sos to the plasma membrane where it activates Ras signaling and stimulates cell growth and proliferation through the PI3K and Raf pathways. Neurofibromin, the gene product of NF1, balances this proliferative pathway by converting GTP to GDP and inactivating Ras. In the absence of normal amounts of neurofibromin, as seen in type 1 neurofibromatosis, Ras activation is unchecked and unregulated cellular growth and tumor formation can occur. It would be of interest to learn if drugs that specifically inhibit PDL2, such as raloxofine, might play a useful therapeutic role in neurofibromatosis patients who have elevated *ARF4* expression.

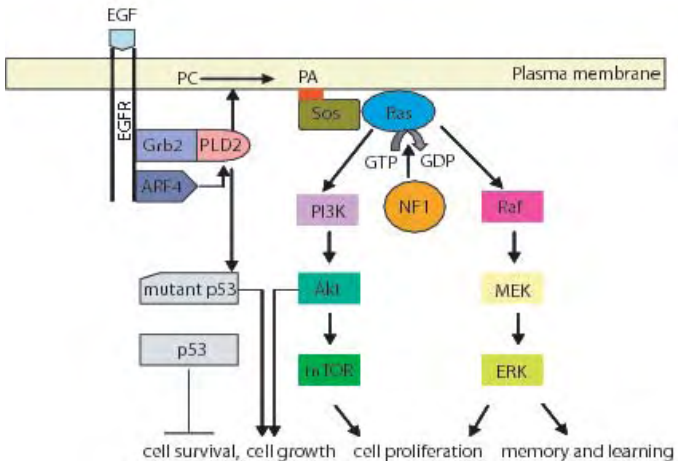

Figure 4. Simplified model of EGFR/Ras signaling pathway in human cells.
ARF4 can bind with EGFR and activate PLD2. The phosphatidic acid (PA) produced by PLD2 can recruit Sos, which can then colocalize and activate Ras signaling. However, NF1 constrains Ras activity through hydrolysis of GTP to GDP. Therefore, both increased ARF4 expression and loss of NF1 expression can lead to elevated Ras activity and enhanced cell proliferation and tumorigenesis. PLD2 can independently stabilize mutant p53 providing a cell survival signal allowing the cell to pass through a checkpoint of the cell cycle, leading to cell proliferation and potential tumorigenesis.

KEY RESEARCH ACCOMPLISHMENTS

- When *IGF2* imprinting is disrupted, its long range interactions change dramatically.
- *ARF4* transcription appears to be dysregulated in neurofibromatosis cell line and specimens, with increased gene expression in subjects with sever disease. *ARF4* might play a role in neurofibromatosis 1 tumorigenesis.
- CTCF is an important regulator of long range interactions.

REPORTABLE OUTCOMES

No new publications during this reporting period

CONCLUSIONS

1. When mutations in *NF1* occur, these interactions may be altered, leading to changes in gene expression.
2. *ARF4* might play a role in neurofibromatosis 1 tumorigenesis.
3. The relevance of these gene interactions in regard to the clinical manifestations of neurofibromatosis 1 needs to be investigated.
4. The search for novel remote gene interactions with *NF1* promises to open up totally new ranges of therapeutic targets.

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APPENDICES: none